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Inefficient Formation of a Complex among CXCR4, CD4 and gp120 in U937 Clones Resistant to X4 gp120–gp41-Mediated Fusion

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Certain subclones (designated as minus clones) of the promonocytic U937 cell line do not support efficient infection and fusion mediated by T cell line adapted (TCLA) X4 HIV-1 gp120–gp41 (Env) although the CXCR4 and CD4 concentrations at their surfaces are similar to those at the surfaces of clones susceptible to HIV-1 entry (plus clones) (H. Moriuchi *et al., J. Virol.* **71**, 9664–9671, 1997). To test the hypothesis that inefficient formation of gp120–CD4–CXCR4 complexes could contribute to the mechanism of resistance to Env-mediated fusion in the minus clones, we incubated plus and minus cells with HIV-1 LAI gp120 and coimmunoprecipitated CD4 by using anti-CXCR4 antibodies. The gp120 induced inefficient coimmunoprecipitation of CD4 in the minus clones but not in the plus ones. Overexpression of CD4 resulted in significant restoration of the minus clones' susceptibility to fusion in parallel with an increase in the amount of the gp120–CD4–CXCR4 complexes. These results not only suggest that the resistance to TCLA X4 HIV-1 entry in the U937 minus clones is due to the inability of these cells to efficiently form complexes among CD4, gp120, and CXCR4, but also provide a direct evidence for the correlation between fusion and the cell surface concentration of the complexes among CXCR4, CD4, and gp120. These data and similar recent observations in macrophages suggest that inefficient complex formation among CXCR4, CD4, and gp120 could be a general mechanism of cell resistance to gp120–gp41-mediated fusion and a major determinant of HIV-1 evolution *in vivo*.

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INTRODUCTION

The human immunodeficiency virus (HIV) enters cells by binding its envelope glycoprotein (Env) to the primary receptor CD4 and a coreceptor molecule (reviewed in Dimitrov, 1997; Dimitrov and Broder, 1997)). It has been previously reported that certain subclones of the promonocytic U937 cell line (minus clones) do not support infection and fusion of TCLA X4 HIV-1 strains (Franzoso *et al.*, 1994; Moriuchi *et al.*, 1997a). Based on previous observations that gp120 from TCLA X4 HIV-1 Envs forms a complex with CD4 and CXCR4 (Dimitrov *et al.*, 1998; Lapham *et al.*, 1996; Xiao *et al.*, 1999), we hypothesized that the mechanism of resistance to HIV-1 entry in these cells is caused

by inefficient formation of complexes between CXCR4, CD4, and gp120. Recently, we found that in macrophages CXCR4 does not form observable complexes with CD4 even in the presence of gp120 unlike in monocytes and primary lymphocytes (Dimitrov *et al.*, 1999). Here we present data demonstrating that the resistance to TCLA X4 HIV-1 Env-mediated fusion of U937 minus clones parallels their inability to efficiently form gp120–CD4–CXCR4 complexes and propose that inefficient complex formation could be a general mechanism of cell resistance to gp120–gp41-mediated fusion and a major determinant of HIV-1 evolution *in vivo*.

MATERIALS AND METHODS

Cells, vaccinia viruses, gp120, sCD4, and antibodies. The U937 subclones 10 and 30 (plus clones) and 17 and 34 (minus clones) were previously described (Franzoso *et al.* 1994). The cell line, TF228, which constitutively expresses HIV LAI Env was provided by Z. Jonak (SmithKline Beecham Pharmaceuticals, Philadelphia, PA). The vaccinia virus (vCB3) encoding the gene for CD4 was previously described (Broder *et al.*, 1993). The HIV LAI gp120 was produced by coinfection of BS-S-1 cells (ATCC CCL26) with vaccinia virus recombinant vPE6 via the hybrid vaccinia virus-T7 system (Fuerst *et al.*, 1986) with a multiplicity of infection of 10 PFU/cell under serum-free medium (OPTI-MEM, Life Technologies, Gaithersburg, MD) conditions and purified from the culture supernatants 30 h postinfection by affinity chromatography using lentil lectin–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (Earl *et al.*, 1994). The gp120 was visualized by SDS–PAGE (10% gel) with Coomassie blue G250 and quantitated by image analysis with the Eagle Eye II (Stratagene, La Jolla, CA) by comparison to a previously prepared reference standard of gp120 purified under identical conditions and quantitated by amino acid analysis (P. Earl, personal communication). Soluble CD4 (sCD4) and the anti-CD4 polyclonal antibody T4-4 were obtained through the AIDS Research and Reference Reagent Program from R. Sweet (SmithKline Beecham Pharmaceuticals). The anti-CD4 mAb OKT4 was purchased from Ortho Diagnostic (Raritan, NJ). The anti-CXCR4 mAbs m171, m172, and m173 were purchased from R&D Systems (Minneapolis, MN). The anti-CXCR4 mAb 12G5 was a kind gift from J. Hoxie (University of Pennsylvania, Philadelphia, PA). The anti-CXCR4 mAb 4G10 and the rabbit polyclonal antibody R2225 were raised to peptides corresponding to regions of the CXCR4 N-terminus and the second extracellular loop, respectively.

Immunoprecipitation. Cells (5×10^6 per sample) were washed once with phosphate-buffered saline (PBS), labeled with biotin if needed, and then resuspended in PBS at a final concentration of 10^7/ml. Immunoprecipitating antibodies (3 μg/ml) were added to the cell suspension and incubated with gentle mixing for 1 h at 37°C. Cells were then pelleted by centrifugation and resuspended in lysis buffer (1% Brij97, 5 mM iodoacetamide (added immediately before use), 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA, and protease inhibitors) at 4°C for 1 h with gentle mixing. The cell lysate was centrifugated at 14,000 rpm for 25 min in a refrigerated Eppendorf centrifuge. Protein G–Sepharose beads (Sigma, St. Louis, MO) (20 μl) prewashed with PBS were added to the supernatant and incubated at 4°C for 14 h. The beads were washed four times with 1 ml of ice-cold lysis buffer. The samples were then eluted by adding 4× sample buffer for SDS–PAGE gel and boiling for 5 min. After electrophoresis in a 10% SDS–PAGE gel they were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked in 20 mM Tris–HCl (pH 7.6) buffer containing 140 mM NaCl, 0.1% Tween-20, and 5% nonfat milk. For Western blotting these membranes were incubated with the respective antibodies and then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. For detection of cell surface biotinylated proteins the nitrocellulose membranes were incubated with streptavidin-conjugated HRP. In both cases they were developed by using the Supersignal chemiluminescent substrate from Pierce (Rockford, Il). Silver staining was performed by using the Silver Stain Plus kit following the company protocol (Bio-Rad Laboratories, Hercules, CA).

Flow cytometry. Cells (5×10^4) were incubated for 1 h on ice with the respective antibodies (10 μg/ml), washed, and incubated for another hour on ice with rabbit IgG (10 μg/ml) (Sigma) to reduce nonspecific binding. The cells were then washed and incubated with an anti-mouse phycoerythrin (PE) -conjugated polyclonal antibody (Sigma) for 1 h, followed by washing and fixation with paraformaldehyde on ice for 10 min. The PE fluorescence of each cell was measured by flow cytometry with a FACSCaliber (Becton–Dickinson, San Jose, CA). The number of receptor molecules was estimated from the signal intensity by using an equation describing the intensity of calibrating beads with a known number of PE molecules (Becton–Dickinson). It was assumed that each antibody molecule is conjugated on average with two PE molecules and binds to one molecule primary antibody which is bound to two receptor molecules thus suggesting a 1:1 ratio of the PE:receptor molecules.

Although these assumptions may not be entirely quantitatively correct, the stochiometry of associated secondary–primary antibodies and primary antibody–receptor molecules may not significantly affect the estimated relative number of receptors at the surfaces of different cells.

Cell fusion assay. To measure fusion we used both a syncytium assay (Dimitrov *et al.*, 1991) and a reporter gene enzyme (β-gal) (Nussbaum *et al.*, 1994). Equal numbers (10^5) of target cells (U937 clones) and effector cells (TF228) were mixed in a 96-well plate and kept at 37°C. Fusion by the β-gal assay was measured 2 h later (4 h when CD4 was not overexpressed) and syncytia were counted 12 h after mixing the cells. Pictures were taken by a video imaging system based on an Olympus IX70 microscope coupled to a CCD camera (Princeton Instruments, Trenton, NJ) and Metamorph software (Universal Imaging Corporation, West Chester, PA).

RESULTS

Inefficient induction of CD4–CXCR4 coimmunoprecipitation by gp120 in U937 minus clones. It has been previously shown that CXCR4 can form a complex with gp120 and CD4 in cell lines susceptible to X4 HIV-1 Env-mediated fusion and proposed that this complex plays a critical role in the initial stages of HIV-1 entry (Dimitrov, 1996; Dimitrov *et al.*, 1998; Lapham *et al.*, 1996; Xiao *et al.*, 1999). To determine whether the formation of the CXCR4–CD4–gp120 complex is differentially affected in the minus U937 clones compared to the plus ones, we incubated both types of cells with gp120 from the HIV LAI strain at 37°C and immunoprecipitated surface-associated CXC4 by using two different anti-CXCR4 mAbs (m173 and 4G10). We found that CD4 was more efficiently coimmunoprecipitated in the plus clones than in the minus ones (Fig. 1A). CD4 associated with CXCR4 even without gp120 although at lower efficiency. Similar results were obtained from experiments performed at 4 and 20°C (data not shown). The coimmunoprecipitated CD4 was quantitated by using serial dilutions of soluble CD4 molecules for calibration (Fig. 1B). In the plus clones the amount of coimmunoprecipitated CD4 induced by gp120 was 3 to 10-fold greater than in the minus clones (Figs. 1A and 2C and data not shown). Similar to what was shown before by using flow cytometry (Moriuchi *et al.*, 1997b) we found that the CD4 surface concentration in minus cells was equivalent or higher than in plus cells (Fig. 1C) indicating that a lower CD4 concentration is not a likely explanation for the low efficiency of gp120–CD4–CXCR4 complex formation in the minus cells. Similar results were obtained for another pair of minus (clone 34) and plus (clone 30) cells (Fig. 1C).

Restoration of the gp120–CD4–CXCR4 complex formation and the fusion ability of the U937 minus clones by overexpression of CD4. The observation that the gp120–

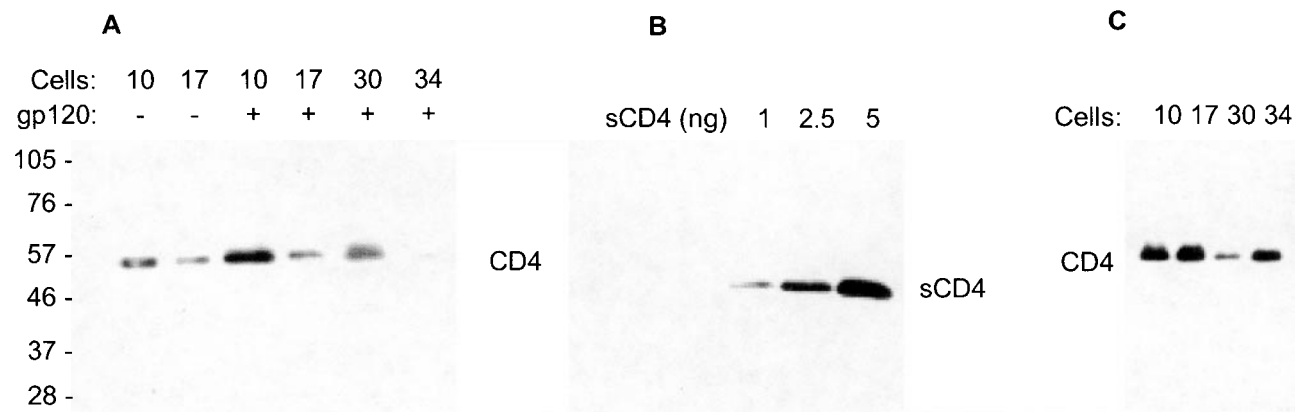

FIG. 1. CD4 coimmunoprecipitation by anti-CXCR4 mAbs. (A) CD4 is efficiently coimmunoprecipitated by an anti-CXCR4 mAb (m173) after incubation with gp120 in plus cells (U937 clone 10) but weakly in minus cells (U937 clone 17) (+gp120). Even in the absence of gp120 CD4 is coimmunoprecipitated although weakly (−gp120). Cells (2×10^7 of each type) were used and the interaction was measured at 37°C . CD4 was detected by Western blotting using the polyclonal antibody T4-4. (B) Calibration of the amount of CD4 by using serial dilutions of sCD4 (5, 2.5, and 1 ng) and following the same procedure as in (A). (C) The level of surface-associated CD4 immunoprecipitated by the anti-CD4 mAb OKT4 from minus cells is comparable to that from plus cells. The cells from each type (1.4×10^7) were first surface labeled with biotin and then lysed and immunoprecipitated with the anti-CD4 mAb OKT4.

CD4–CXCR4 complex formation in the minus clones is inhibited but not completely blocked suggested that it could be restored by increasing the concentration of the components forming this complex. Therefore, we overexpressed CD4 by using vaccinia virus and found that in both types of cells the amount of coimmunoprecipitated CD4 is significantly increased (Fig. 2). At these high CD4 concentrations the number of the gp120–CD4–CXCR4 complexes and the number of coimmunoprecipitated CD4 molecules induced by gp120 were about the same for the plus and minus clones (Figs. 2B, and 2C). We also observed an increase in the amount of CXCR4 associated with CD4 even in the absence of gp120, although the addition of gp120 significantly increased this association (Fig. 2). The restoration of the gp120–CD4–CXCR4 complex formation in the minus clones paralleled an increase of their fusion ability. Minus cells fused with TF228 cells expressing the HIV-1 Env (LAI) at nearly the same efficiency as the plus clones after 12 h of incubation (Fig. 3). These results indicate that a major determinant of the resistance to HIV Env-mediated fusion in the minus clones is related to their inability to support efficient gp120–CD4–CXCR4 complex formation.

Correlation between fusion and concentrations of gp120–CD4–CXCR4 complexes. The observation that increases in fusion parallel increases in complex formation suggested possible correlations. By using a phosphoimager for quantitation (Figs. 1 and 2) we found that the number of gp120–CD4–CXCR4 complexes correlated significantly with fusion efficiency ($P=0.003$). The data were fitted by a linear regression (Fig. 4) suggesting direct proportionality between the number of complexes and fusion events.

Evaluation of the CXCR4 concentration and epitope structure at the surface of the U937 plus and minus cells by a panel of mAbs. The inability of the minus cells to efficiently form gp120–CD4–CXCR4 complexes could be due to low concentrations or structural changes of CXCR4 when expressed in the minus clones that may interfere with the CXCR4–CD4–gp120 interactions. It was found in a previous study (Moriuchi *et al.*, 1997a) that both CXCR4 and CD4 are expressed at the surface of the plus and minus cells at similar concentrations but their surface concentrations were not quantitated and only one mAb (12G5), which is sensitive to conformational epitopes, was used for the flow cytometry detection of CXCR4. It was possible that the actual surface concentration of CXCR4 is significantly higher for the plus clones compared to the minus ones and that could explain their differential resistance to HIV-1 entry. To investigate this question and also to find possible differences in the antigenic structure of CXCR4 which could contribute to their differential interactions we tested several

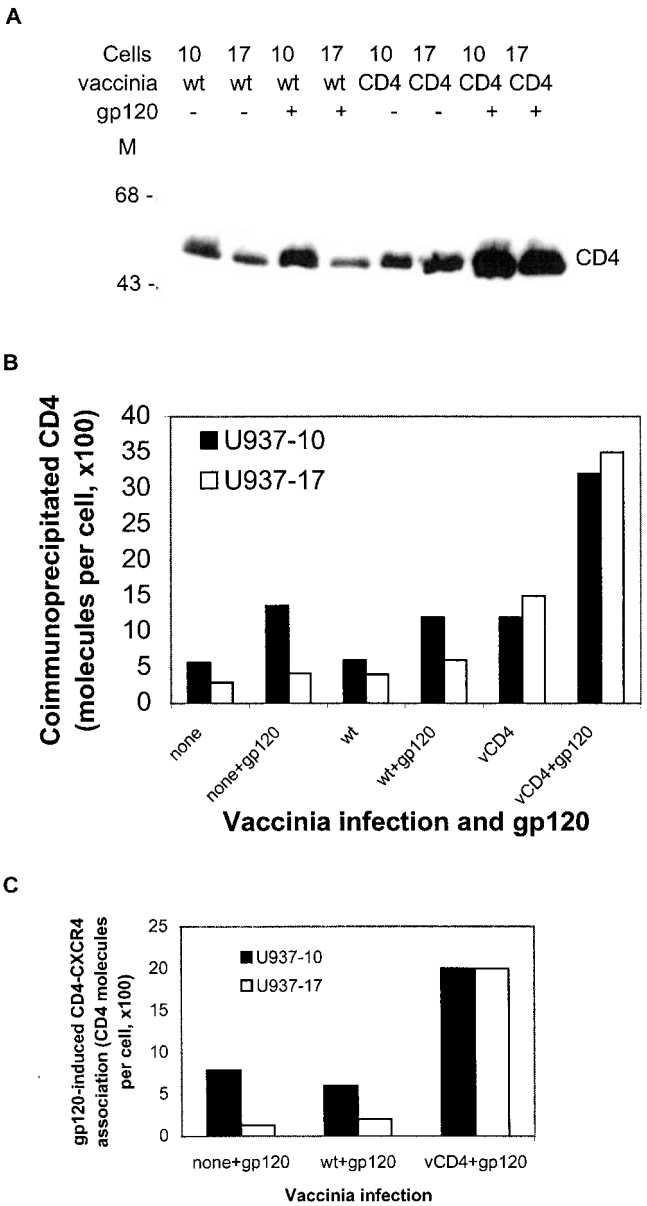

FIG. 2. Coimmunoprecipitated CD4 increases with an increase in the amount of CD4 overexpressed by a recombinant vaccinia virus in plus and minus cells. (A) Western blotting with an anti-CD4 antibody (T4-4) after SDS–PAGE of cells infected with either wild-type (wt) vaccinia virus (WR) or a recombinant vaccinia virus (vCB3) containing the gene for CD4 (CD4). Cells (2×10^7 of each type) were used and the interaction was measured at 37 °C in presence (+gp120) or absence of gp120 (−gp120). Infection with the vaccinia viruses was performed as previously described (Broder *et al.*, 1993). (B) Quantitation of the number of coimmunoprecipitated CD4 molecules. The images were acquired with a Bio-Rad phosphoimager (Bio-Rad, Hercules, CA) and the signal intensity was measured by the program Molecular Analyst (Bio-Rad). The number of molecules was then calculated from the calibration images of sCD4 with known number of molecules (an example is shown in Fig. 1B). (C) Coimmunoprecipitated CD4 induced by gp120. The number of coimmunoprecipitated CD4 molecules in the absence of gp120 was subtracted from that in the presence of gp120.

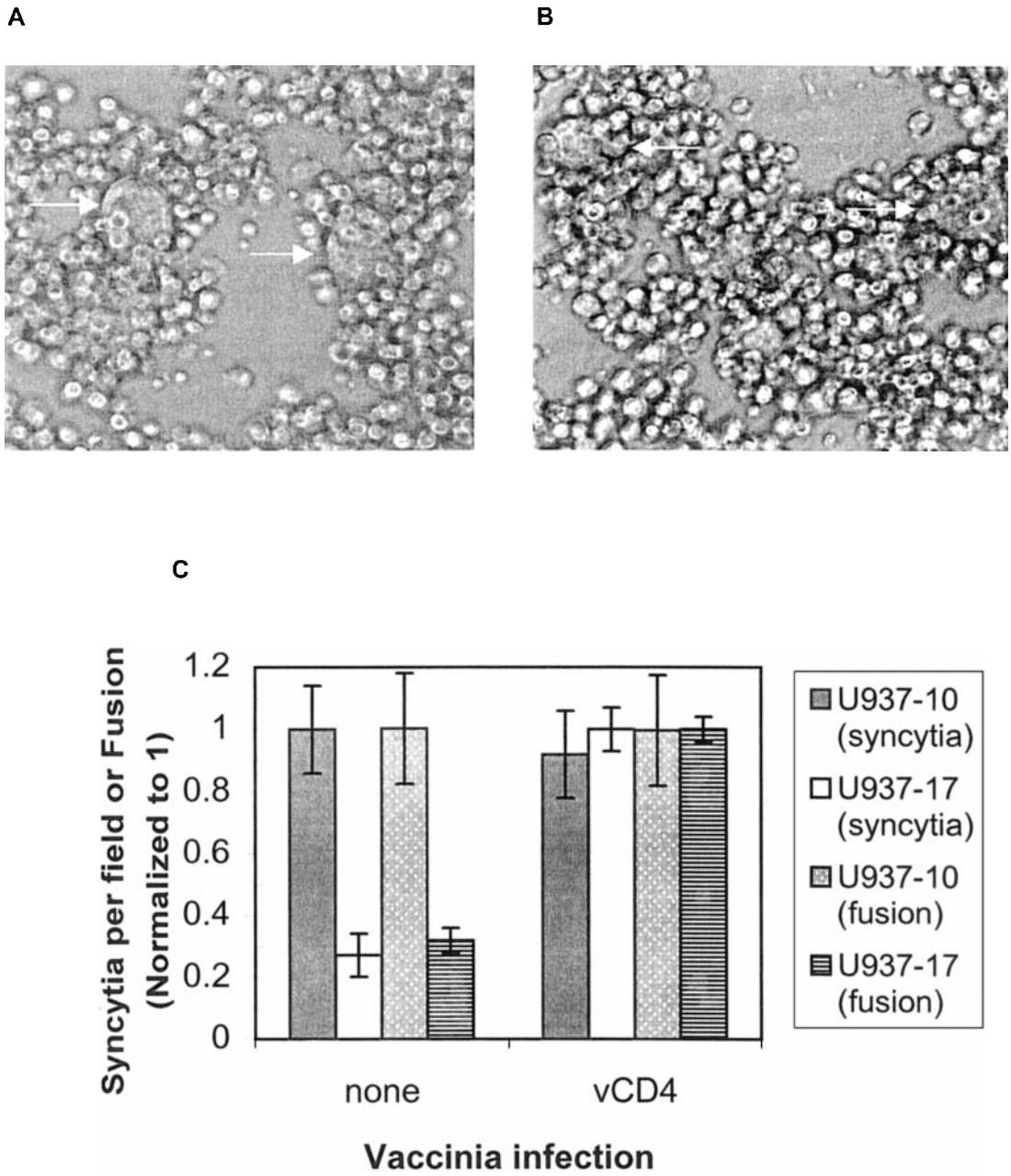

FIG. 3. Overexpression of CD4 in minus cells leads to restoration of their fusion ability with cells expressing TCLA X4 HIV-1 Env. Plus cells (U937-10) (A) or minus (U937-17) (B) were mixed with equal numbers (5×10^4) of TF228 cells expressing the HIV-1 LAI Env and pictures were taken 12 h later. The arrows denote syncytia. (C) Quantitation of the number of syncytia per field of observation. The T lymphocytic cell line CEM was used as a positive control.

anti-CXCR4 mAbs for their ability to bind to the plus and minus clones. We observed high nonspecific binding of the mouse mAbs to the surface of the minus clones (17, 34) but not to the plus clones (10, 34) and also noted that rabbit IgG cross-reacted with the binding sites for the mouse IgG. We were not able to identify the molecules which were involved in the mouse and rabbit IgG binding and could account for the resistance of the minus clones to HIV-1

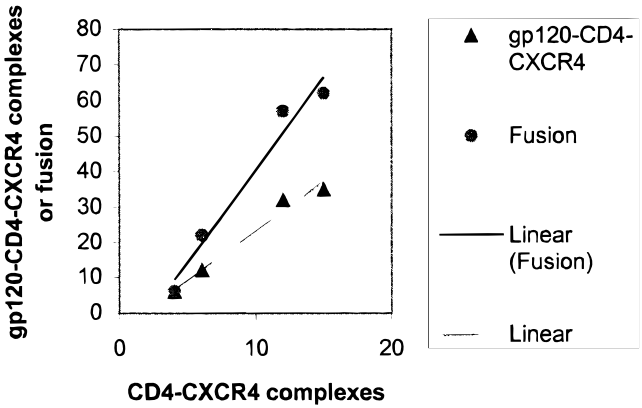

FIG. 4. Correlation between the amount of CD4 associated with CXCR4 in the absence of gp120 (CD4–CXCR4), in the presence of gp120 (gp120–CD4–CXCR4), and cell fusion. The quantitation of the number of coimmunoprecipitated CD4 molecules was performed as described in the legend to Fig. 2 and cell fusion was determined as the number of syncytia per field of observation. The linear regression analysis lines were obtained by using the program Excel.

entry. However, this observation was used to eliminate the background due to nonspecific mouse mAb binding and in the subsequent experiments the cells were preincubated with rabbit IgG which allowed a more accurate estimation of the number of CXCR4 and CD4 molecules at the surfaces of the two types of cells.

Figure 5 presents examples from the results obtained by a flow cytometry analysis using anti-CXCR4 mAbs. The estimation of the number of molecules at the cell surface using calibration beads suggested that the plus clones express about 2×10^4 molecules per cell while the minus clones express about 3×10^4 molecules per cell. However, the diameter of the minus clones is approximately about 1.3 times larger than that of the plus clones and therefore the

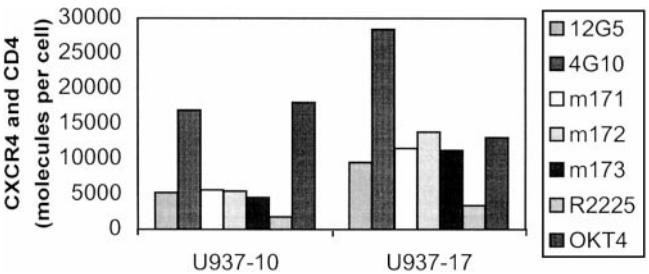

FIG. 5. Evaluation of CXCR4 surface concentration and antigenic structure by flow cytometry. Estimation of the number of CXCR4 and CD4 molecules at the surface of plus (U937-10) and minus (U937-17) cells.

average surface concentration of CXCR4 in both types of cells is approximately the same. One should note that the real surface concentrations of CXCR4 could be lower because several molecules of the secondary antibody could bind to one molecule of the primary anti-CXCR4 antibody. However, the important observation was that the ratios of the amounts of bound mAbs to the minus cells to those bound to the plus cells were about the same. This suggests that the epitopes of these mAbs are equally well recognized in the two types of cells, and there are no differences in their exposure which would account for the inhibition of HIV entry into the minus cells. This observation does not rule out the possibility that other epitopes not recognized by these mAbs are different.

DISCUSSION

A major finding of this work is that the mechanism of resistance to TCLA X4 HIV-1 Env-mediated fusion of promonocytic cell clones is related to inefficient formation of CXCR4–CD4–gp120 complexes. This result is important because it suggests a likely and possibly universal mechanism explaining a number of observations that expression of CD4 and HIV coreceptors is not sufficient for HIV entry into cells. Two recent reports support this notion suggesting that a similar mechanism operates for TCLA X4 HIV-1 Env-mediated fusion of macrophages (Dimitrov *et al.*, 1999; Lapham *et al.*, 1999). Further, it directly demonstrates a correlation between the ability of the HIV-1 Env to mediate membrane fusion and the formation of the CXCR4–CD4–gp120 complex as an initial stage of HIV-1 entry. Such a mechanism has been proposed by several investigators (Dimitrov, 1996; Golding *et al.*, 1995; Lapham *et al.*, 1996; Trkola *et al.*, 1996; Wu *et al.*, 1996) but has not been formally experimentally demonstrated.

The molecular mechanism accounting for the inability of the minus clones to efficiently form gp120–CD4–CXCR4 complexes is still unclear. In cell types expressing equivalent amounts of CXCR4 and CCR5 and a low number of CD4 molecules a scenario of competition between CXCR4 and CCR5 for association to CD4 may influence HIV-1 Env-mediated fusion, as suggested for primary macrophages (Broder and Dimitrov, 1996; Dimitrov *et al.*, 1999). However, the significant expression of CD4 and CXCR4 in the minus clones makes this model an unlikely explanation. Alternatively, poor fusion and complex formation could be related to the existence of modified CXCR4 which is not

able to interact efficiently with the CD4–gp120 complex. The significant overexpression of CD4 by vaccinia virus may overcome this "defect" or alteration in CD4 association with a modified CXCR4. Currently we are trying to characterize different forms of CXCR4 in their ability to support complex formation. Another possibility is that CD4 in the minus clones could be inhibited in its ability to interact appropriately with CXCR4 either directly or by an inhibitory molecule(s). Since vaccinia virus infection inhibits host cell protein synthesis, there may not be enough of the putative inhibitory molecule(s) to interact with the overexpressed CD4 receptor. We also cannot exclude the possibility that the endogenous CD4 is differentially modified as well. Although after 12 h of incubation the level of syncytia formation between X4 HIV-1 Env-expressing cells and U-937 plus and minus clones infected with vaccinia virus encoding CD4 is similar, kinetic studies have revealed a difference in the extent of fusion during early times (2 h) and suggests that other factors may be influencing the clustering of sufficient CD4–CXCR4 complexes for fusion pore formation (data not shown).

Whatever the specific molecular mechanism of the inefficient complex formation in the minus clones is, the overexpression of CD4 increases the number of CXCR4 molecules which are associated with CD4 and therefore the number of gp120–CD4–CXCR4 complexes, resulting in fusion. This interpretation is consistent with the elegant studies demonstrating the role of CD4, CXCR4, and CCR5 surface concentrations for fusion of HeLa cell lines (Kozak *et al.*, 1997; Platt *et al.*, 1998).

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